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Immunological Characterization of Urinary 8-Epi-Prostaglandin $F_{2\alpha}$ Excretion in Man[1]

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ABSTRACT

F_2-isoprostanes are prostaglandin (PG) F_2-like compounds that are formed *in vivo* directly by free radical-catalyzed lipid peroxidation. One of the compounds that can be produced in abundance by such mechanism is 8-epi-$PGF_{2\alpha}$, a potent vasoconstrictor. We have developed an enzyme immunoassay and a radioimmunoassay for measuring urinary concentrations of 8-epi-$PGF_{2\alpha}$ by raising antibodies against this compound. The antisera presented high titers (>1/300,000) and provided highly sensitive assays (IC_{50}, 8 and 24 pg/ml, for EIA and RIA, respectively); cross-reactivity with other PG was negligible. The interassay reproducibility of EIA was assessed by measuring the same urine stored frozen in aliquots after solid phase extraction and thin-layer chromatography (17%, $n = 13$). Measurements of urinary 8-epi-$PGF_{2\alpha}$ by immunoassays were validated using different antisera and by comparison with gas chromatography/mass spectrometry. Healthy volunteers excreted 25 ± 12 ng of 8-epi-$PGF_{2\alpha}$/mmol creatinine ($n = 19$), with no circadian variation over three consecutive 8-hr collection periods ($n = 10$); preliminary results showed that excretion increased as a function of age. Urinary excretion of 8-epi-$PGF_{2\alpha}$ was unchanged by treatment with two nonsteroidal antiinflammatory drugs, ibuprofen at 1.2 g/day for 4 days ($n = 4$) or aspirin as a single administration of 1 g ($n = 6$). In contrast, the urinary excretion of 11-dehydro-thromboxane B_2, a platelet cyclooxygenase-derived metabolite was reduced by more than 80% after aspirin administration. Analysis of serum revealed a small (0.1% of thromboxane B_2) but consistent production of 8-epi-$PGF_{2\alpha}$ by a cyclooxygenase-dependent mechanism totally suppressed after administration of aspirin to the same subjects. Monitoring of this compound in urine or plasma may turn to be a useful index of *in vivo* lipid peroxidation.

Lipid peroxides have been reported to accumulate in certain diseases as a consequence of free-radical attack occurring in advanced atherosclerosis, stroke and myocardial infarction. Recently, a series of PG F_2-like compounds termed F_2-isoprostanes produced *in vivo* in humans was discovered. These compounds are formed by a noncyclooxygenase, free radical-catalyzed mechanism involving peroxidation of arachidonic acid (Morrow *et al.*, 1990a and b). One of the compounds, 8-epi-$PGF_{2\alpha}$ (fig. 1) has attracted attention because of its biological activity; it is a potent vasoconstrictor in the rat and its action has been shown to be mediated at least in part via interactions with vascular TXA_2/PGH_2 receptor (Takahashi *et al.*, 1992). In addition, this compound behaves as a partial agonist on platelet aggregation because it induces platelet shape change but acts as an antagonist of TXA_2/PGH_2 receptors on both human and rat platelets when platelet aggregation is induced by TXA_2 mimetics (Hecker *et al.*, 1987; Morrow *et al.*, 1992c). In contrast to lipid hydroperoxides that decompose rapidly in human fluids or tissues, F_2-isoprostanes are chemically stable end-products of lipid peroxidation. These compounds can be formed from arachidonic acid in the phospholipids and subsequently be released by phospholipases, preformed. They are present in both plasma and urine estimated by GC/MS measurement although the internal standard used in these studies was not itself an isoprostane (Morrow *et al.*, 1990b; Morrow *et al.*, 1992a and Morrow *et al.*, 1992b). Measurement of 8-epi-

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ABBREVIATIONS: EIA, enzyme immunoassay; TX, thromboxane; RIA, radioimmunoassay; PG, prostaglandin; TLC, thin layer chromatography; NICI-GC/MS, negative ion chemical ionization-gas chromatography/mass spectrometry.

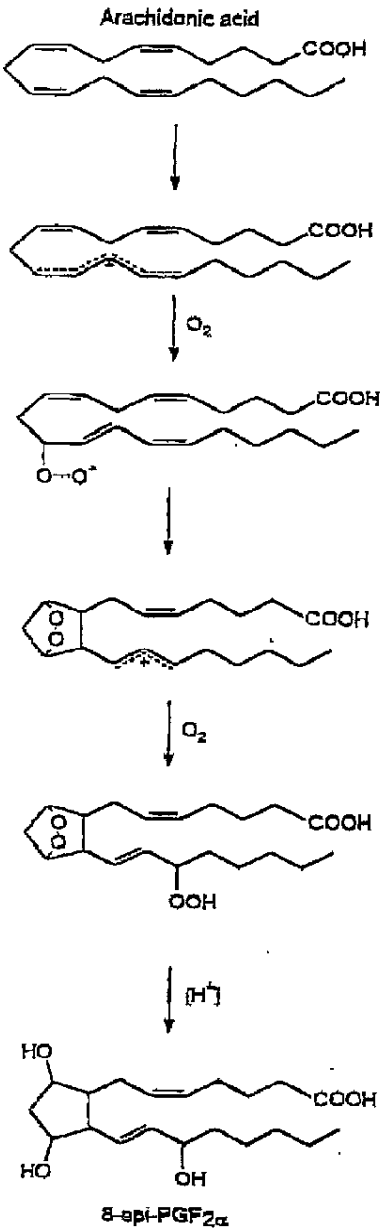

Fig. 1. Free radical transformation of arachidonic acid into 8-epi-$PGF_{2\alpha}$ (from Morrow *et al.*, 1990b)

$PGF_{2\alpha}$ as a marker of oxidant injury may provide a unique index to reflect endogenous lipid peroxidation and/or monitor the formation of a potent biologically active compound. The aim of this study was to obtain antibodies against 8-epi-$PGF_{2\alpha}$ and subsequently develop sensitive immunoassays that enable the determination of this compound in complex biological fluids such as serum or urine.

Materials and Methods

Materials and preparation of antisera. 8-epi-$PGF_{2\alpha}$ and other PG standards were purchased from Cayman Chemicals, Ann Arbor, MI. [^{3}H]-8-epi-$PGF_{2\alpha}$ (28.4 Ci/mmol) was a kind gift of Dr. Kamal Badr. 8-Epi-PGE_2 was a kind gift of Dr. L. J. Roberts II. Keyhole limpet hemocyanin (KLH), 3.5% phosphomolybdic acid solution, 5-hydroxy-tempo, butylated hydroxy-toluene (BHT), human and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO. (5,6,8,9,11,12,14,15-^{3}H(N)-TXB_2 3.7–3.25 Tbq/mmol) was obtained from New England Nuclear Du Pont, Paris, France. Baker Bond solid phase extraction cartridges, all solvents high performance liquid chromatography grade were from Baker, Phillibsburg, N.J. TLC plates (Silicagel G 20 × 20 cm) were from Merck Darmstadt, Germany. Antisera were obtained using two different approaches. Coupling of 8-epi-$PGF_{2\alpha}$ to keyhole limpet hemocyanin was achieved as described earlier (Pradelles *et al.*, 1985) and the immunization protocol essentially followed that of Vaitukaitis *et al.* (1971). Alternatively, 8-epi-$PGF_{2\alpha}$ was conjugated to human serum albumin using the carbodiimide method and a previously established protocol (Ciabattoni, 1987).

Urine collection in healthy subjects. The samples were collected throughout a 6- to 8-mo period from 19 healthy subjects (12 males, 7 females, nonsmokers and 2 smokers, age range 21–54 yr) who had not taken any drugs for 15 days before urine collection, unless specified. In a preliminary attempt to assess for the influence of increasing age, another group of 20 healthy volunteers (nonsmokers, aged 21–77 yr) was also investigated. Overnight urines (approximately 8–10 hr) were collected in the morning, fractionated in 50-ml aliquots with 5-hydroxy-tempo, a free radical scavenger and EDTA, 1 mM final each (Sigma) and stored at −70°C until analysis. An aliquot was stored without the antioxidant for creatinine measurement by the Jaffe chromogen method. Separately, the antioxidant was added to a single urine sample immediately after voiding. It was then divided into six 10-ml aliquots and frozen immediately or after 1, 2, 3, 4 or 5 h storage at room temperature. Analysis of 8-epi-$PGF_{2\alpha}$ by RIA (see later) did not show any peculiar trend with time, suggesting that under these conditions, no spontaneous formation or degradation of this compound occured. In another setting, a large volume of urine to which was added 5-hydroxy-tempo/EDTA was divided into aliquots and stored at −70°C. Analysis of 8-epi-$PGF_{2\alpha}$ over a 6-mo period did not show any variation of the levels suggesting that this precaution prevented *ex vivo* autoxidation reported earlier (Morrow *et al.*, 1990a). For this purpose, samples with high and low values (120–627 pg/ml urine) stored at −20°C, analyzed at 6-mo interval did not show any significant variation of values (353 ± 194 vs. 350 ± 222 pg/ml urine, $n = 6$, Mean ± SD with less than 10% difference in value for each individual samples). To assess whether circadian variation in F_2-isoprostane occurs, urine was collected over 24 hr in 10 subjects as three consecutive 8-hr samples and was then stored as described above. Finally to determine if cyclooxygenase inhibition can alter urinary F_2-isoprostanes, six healthy subjects (six males) ingested 1 g aspirin (lysine salt) at 6 P.M. and three 8-hr urine collections were obtained as mentioned above, starting at 1 A.M. the next day. Ibuprofen was administered (1.2 g daily) orally for 5 consecutive days to four healthy subjects (two males, two females) and urine was collected before and during the period of drug administration.

Serum. TXB_2 production during whole blood clotting before and after aspirin ingestion was measured in five of the six subjects mentioned above (Patrono *et al.*, 1980). The anti-TXB_2 and the anti-11-dehydro-TXB_2 sera used were obtained in our laboratory (Pradelles *et al.*, 1985; Ciabattoni *et al.*, 1989). After coagulation, BHT was added to the serum samples as anti-oxidant and the samples were immediately frozen at −70°C until analysis.

EIA analysis. Purification and extraction of urine for EIA analysis was identical to that performed by Lellouche *et al.* (1990) with the following exceptions: 8000 cpm of [^{3}H]-TXB_2 was added to urine

for recovery estimation; Bakerbond cartridges were used for solid phase extraction. Authentic 8-epi-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 were run on a separate lane and visualized using phosphomolybdic acid spray. The zones corresponding to 8-epi-$PGF_{2\alpha}$ (Rf 0.13), TXB_2 (Rf 0.21) and 11-dehydro-TXB_2 (Rf 0.29) were scraped off and eluted with 1 ml EIA buffer (see below). The fraction containing [^{3}H]-TXB_2 was counted to estimate for losses occuring during the purification steps.

On some occasions, serum was analyzed for its 8-epi-$PGF_{2\alpha}$ content by direct assay; results were compared to those obtained after purification of the extracts by TLC. [^{3}H]-TXB_2 was added (see above) and the proteins precipitated overnight after addition of ice-cold methanol (serum/methanol, 1:4, v/v). After centrifugation, the supernatant was diluted with water to reach a final concentration of 1.0% methanol and the samples extracted and run on TLC as described above prior to EIA analysis.

After purification, the different metabolites were measured by EIA using a published procedure (Pradelles *et al.*, 1985). Tracers (8-epi-$PGF_{2\alpha}$, TXB_2 or 11-dehydro-TXB_2 coupled to acetylcholine esterase from the electric eel) and corresponding antisera were then added (50 μl each) at appropriate dilutions. The protocol followed that described by Pradelles *et al.* (1985). Fitting of the standard curves and calculations were done with a microcomputer (IBM) using a linear log-logit transformation (Rodbard and Lawald, 1970). All measurements were done in duplicate. For urines, the results given by the computer (ng/ml) were corrected according to creatinine and recovery (calculated from the addition of [^{3}H]-TXB_2 to urinary samples).

RIA analysis. After adjusting the urine pH to 4.0 with formic acid, 10-ml urine aliquots were extracted on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) and eluted with 10 ml ethyl acetate. The eluates were subjected to silicic acid column chromatography and further eluted with benzene:ethyl acetate:methanol (60:40:30, v/v). Theses eluates were dried, recovered with 5 ml of buffer and assayed in the RIA system at a final dilution ranging between 1:30 to 1:60. Recovery was evaluated by adding 4,000 cpm of [^{3}H]-8-epi-$PGF_{2\alpha}$ or [^{3}H]-TXB_2 to each urine sample before extraction and by counting two 1-ml aliquot of the extracted and purified mixture. The overall recovery from the whole procedure averaged 78.3 ± 6.0% (mean ± S.D., n = 24) for [^{3}H]-8-epi-$PGF_{2\alpha}$ and 77.6 ± 5.1% (n = 28) for [^{3}H]-TXB_2. A single eluate obtained from a urine pool was subjected to reverse-phase high-performance liquid chromatography (C18, 220 × 4.6 mm, 5 μm, Brownlee column) with the solvent system, acetonitrile:water:acetic acid (27:73:0.18) at a flow rate of 0.5 ml/min; 0.5-min samples were collected for 30 min. Each fraction was vacuum-dried in a Speedvac evaporator linked with a Savant-refrigerated condensation trap, recovered with 1 ml buffer and tested in the RIA system. The peak of 8-epi-$PGF_{2\alpha}$ eluted at 12 min; it was identified by UV profiling of authentic standard (100 ng). Retention times of other PG were 5 min for 6-keto-$PGF_{1\alpha}$, 12 min for 8-epi-$PGF_{2\alpha}$ and 24.5 min for PGE_2. The peak widths ranged 1 to 2 min.

Approximately 2500 dpm of [^{3}H]-8-epi-$PGF_{2\alpha}$ was mixed with appropriately diluted antiserum in a volume of 1.5 ml of assay buffer (phosphate 0.025 M, pH = 7.5) and incubated for 24 to 30 h at 4°C, to obtain approximately 40 to 45% binding of the labeled hapten. Separation of antibody bound from free [^{3}H]-8-epi-$PGF_{2\alpha}$ was achieved by rapidly adding 0.1 ml of a 5% bovine serum albumin solution and 0.1 ml of a charcoal suspension (70 mg/ml) and subsequent centrifugation at 4°C for 10 min at 5000 rpm (3000 × g). Supernatant solutions containing antibody-bound PG were decanted directly into 10 ml of Instagel (Packard). Radioactivity was counted in a Packard Tri-Carb 1900 CA liquid scintillation counter. Data were processed with the aid of a computer, which was programmed to correct for nonspecific binding.

Negative ion chemical ionization gas chromatography/mass spectrometry analysis (NICI-GC/MS). 8-epi-$PGF_{2\alpha}$ was quantified by a stable isotope dilution assay similar to that previously used for TX metabolites (Catella and FitzGerald, 1987). The internal standard was [$^{18}O_2$] 8-epi-$PGF_{2\alpha}$ produced from authentic 8-epi-$PGF_{2\alpha}$ (Cayman Chemical) using the technique described by Pickett and Murphy (1981). Samples were spiked with 1 ng internal standard and acidified to pH 3 to 3.5 with formic acid. After extraction on a C_{18} solid phase extraction column (Alltech Associates Inc., Deerfield, MI), they were purified by TLC on silica gel plates (LK6D; Whatman Inc., Clifton, NJ) using 90% ethyl acetate/10% methanol/0.1% acetic acid as the mobile phase. The pentafluorobenzyl ester was formed (10% PFB Br in acetonitrile, 10 min at room temperature) and further TLC purification was achieved using ethyl acetate as the mobile phase. The tert-butyldimethylsilyl ether was formed by adding 10 μl N-[tert-butyldimethylsilyl]-N-methyltrifluoroacetamide (MTBSTFA; Sigma) and 10 μl pyridine and allowing the sample to stand at room temperature for 24 hr.

Instrumentation was a Delsi 200 gas chromatograph interfaced with a Nermag Automass 150 mass spectrometer (both from ATI Instruments, Madison, WI). The GC column was a 30 m DB-1 (J&W Scientific, Folsom, CA) programmed from 190 to 320°C at 20°C/min. Helium was the carrier gas. GC/MS interface temperature was 300°C; splitless injection temperature was 260°C. Retention time was approximately 13 min. The mass spectrometer was operated in the negative ion chemical ionization (methane) mode, monitoring m/z 595 for 8-epi-$PGF_{2\alpha}$ and m/z 599 for the [$^{18}O_2$]-labeled internal standard. Integration times were 500 ms for each ion. Quantification was by peak area ratios.

Statistics. Statistics on linear regressions were performed using SigmaStat software (Jandel Co, San Rafaele, CA).

Results

Binding parameters. The best antiserum used for EIA (L#9) was obtained after the 5th booster. It gave a linear response from 2 to 125 pg/ml with an IC_{50} of 8 pg/ml (i.e., concentration required to inhibit initial binding by 50%) as shown from the Log-logit representation (fig. 2); it was used at 1:900,000. For RIA, the best available antiserum (Rab #1) bound 45% of the labeled hapten at a final dilution of 1:200,000. Unlabeled 8-epi-$PGF_{2\alpha}$ displaced the binding of

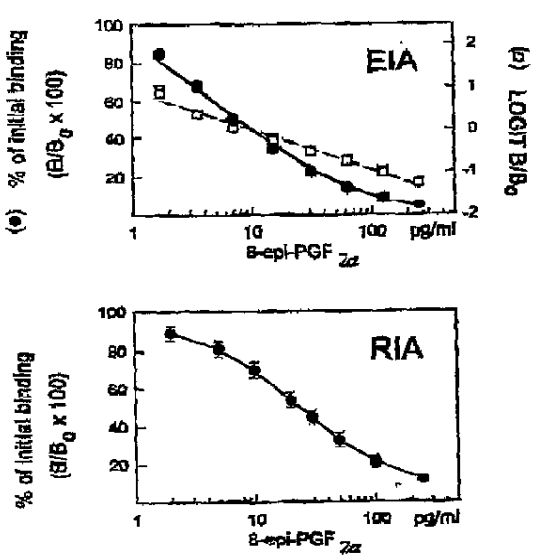

Fig. 2. Dose-response curves of 8-epi-$PGF_{2\alpha}$ immunoassays by EIA (upper panel) or RIA (lower panel). Different antisera (L#9 and Rab#1, respectively) were used in these studies.

the homologous tracer in a linear fashion over the range of 2 to 250 pg/ml, with an IC_{50} of 24 pg/ml (fig. 2). The cross-reactivities with other PG and related metabolites are shown in Table 1. The intra-assay ($n = 6$) and interassay ($n = 8$) coefficients of variation were ± 2.0% and ± 2.9% at the lowest level of standard (2 pg/ml) and ± 3.7% and ± 10.8% at the highest level of standard (250 pg/ml), respectively. The two antisera tested in either immunoassay system expressed a good specificity as evaluated from the limited recognition of heterologous compounds. However, such evaluation does not account for the interference of unrelated substances present in complex biological fluids such as urine or serum. We therefore performed several measurements in biological media to ascertain the specificity of the values obtained with the immunoassays, irrespective of the label.

Validation of the analysis of 8-epi-$PGF_{2\alpha}$ in urine and serum. The reliability of EIA using [^{3}H]-TXB_2 as a recovery tracer was initially verified by spiking known amounts of 8-epi-$PGF_{2\alpha}$ (in excess of the concentration found in the biological matrix) to buffer or to two different volumes of urine. There was a good linearity in the quantification of 8-epi-$PGF_{2\alpha}$ (fig. 3A). The recovery for extraction and further purification of unlabeled 8-epi-$PGF_{2\alpha}$ averaged 73 ± 9% (n = 19) whereas that of [^{3}H]-TXB_2 was on average 54 ± 5%. Confirmation of the validity of this approach was provided by the analysis of increasing volumes of the same pool of urine for which we observed a linear relationship between the volume and the assayed material (fig. 3B).

Final validation of the assay in urine was provided by comparison of values obtained by TLC/EIA with an independent analytical approach, NICI-GC/MS. Different samples of urine were analyzed by GC/MS and the same samples were quantified by EIA following the procedures described in "Materials and Methods." An excellent correlation between the two methods was obtained ($r = 0.99$, $n = 9$, $P < 0.001$; fig. 4A). Additionally, 12 urine samples were extracted and purified for RIA as described in "Materials and Methods." The urine extracts were quantified by RIA using two antisera with a slight difference in cross reactivities (Table I, EIA #9 and RIA #1). Similar values were obtained using either antiserum ($r = 0.99$, $n = 12$ $P < 0.001$; fig. 4B). It should be noted that in both cases, exclusion of the single high ranged 8-epi-$PGF_{2\alpha}$ value still yields correlation coefficients of $r = 0.92$.

TABLE 1

Specificity of anti-8-epi-$PGF_{2\alpha}$ sera used for EIA and RIA

Ligand	Cross-Reactivity of Antisera (%)		
	# 8 EIA	# 9 EIA	# 1 RIA
8-Epi-$PGF_{2\alpha}$	100	100	100
8-Epi-PGE_2	N.D.	0.08	7.7
$PGF_{2\alpha}$	3	0.02	0.24
TXB_2	1.9	0.01	<0.02
6-Keto-$PGF_{1\alpha}$	0.4	<0.01	<0.02
PGE_2	0.01	<0.01	0.56
2,3-Dinor-6-keto-$PGF_{1\alpha}$	1.1	<0.01	<0.01
2,3-Dinor-TXB_2	0.06	0.03	<0.01
PGD_2	1.2	0.2	N.T.
6,15-Diketo-$PGF_{1\alpha}$	N.T.	N.T.	<0.01

Cross reactivities were determined after addition of either homologous (8-epi-$PGF_{2\alpha}$) or heterologous (other PG) ligands to the antibody-tracer complex. Displacement of 50% initial binding was determined for the different compounds and relative % was expressed as the concentration of homologous/concentration of heterologous ligand x 100. N.T., not tested.

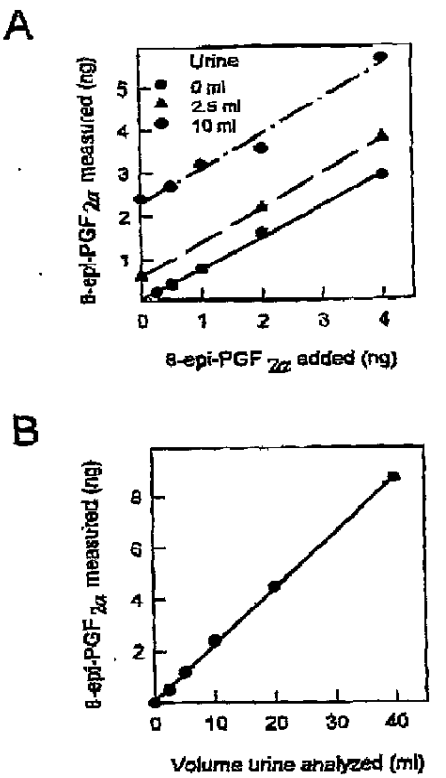

Fig. 3. Recovery and dilution studies of urinary 8-epi-$PGF_{2\alpha}$ by EIA. A, Known amounts of 8-epi-$PGF_{2\alpha}$ were added to buffer or different volumes of human urine (0, 2.5 or 10 ml) and analyzed as described in "Materials and Methods" by EIA. B, Increasing volumes of the same urine were analyzed and the amount of 8-epi-$PGF_{2\alpha}$ determined. [^{3}H]-TXB_2 was added for recovery estimation prior to analysis.

Urinary excretion of 8-epi-$PGF_{2\alpha}$ in normal subjects. Analysis of the daily variation in the excretion of 8-epi-$PGF_{2\alpha}$ in urine was performed in 10 different subjects on a 24 hr period. No statistically significant variation could be observed among the three 8-hr collection periods (fig. 5). The average excretion value of 8-epi-$PGF_{2\alpha}$ was 25 ± 12 ng/mmol creatinine (n = 19) in subjects aged less than 54 yr. However 8-epi-$PGF_{2\alpha}$ excretion significantly correlated ($r = 0.81$; $P < 0.001$) with increasing age (fig. 6).

Because of the non-enzymatic nature of formation of 8-epi-$PGF_{2\alpha}$ (Morrow *et al.*, 1990b) it was of interest to assess the consequences of cyclooxygenase inhibition. We tested the effects of two distinct nonsteroidal antiinflammatory drugs, aspirin and Ibuprofen on the excretion of 8-epi-$PGF_{2\alpha}$ in urine. Single oral dosing with 1 g of aspirin was not associated with any statistically significant variation in the urinary excretion of 8-epi-$PGF_{2\alpha}$ (fig. 7). However, urinary excretion of the platelet-cyclooxygenase-derived 11-dehydro-TXB_2 was inhibited by more than 80%, reflecting the effectiveness of aspirin on its enzymatic target. When the reversible cyclooxygenase inhibitor ibuprofen (400 mg t.i.d.) was administered repeatedly during 4 days, there was no statistically significant change in the excretion of 8-epi-

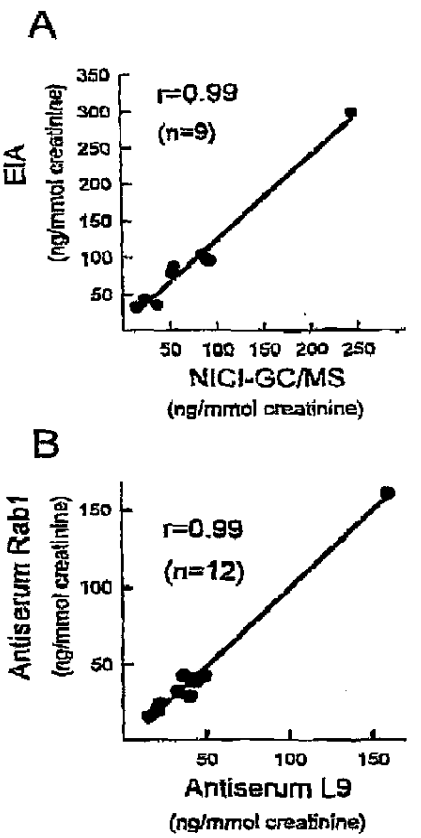

Fig. 4. Validation of immunological measurements of urinary 8-epi-$PGF_{2\alpha}$. A, Correlation between excretion rates of 8-epi-$PGF_{2\alpha}$ as measured in the same urine samples by EIA and NICI GC/MS. B, Correlation between measurements of urine extracts by RIA using two different antisera directed against 8-epi-$PGF_{2\alpha}$. Urines were extracted and purified as described in "Materials and Methods."

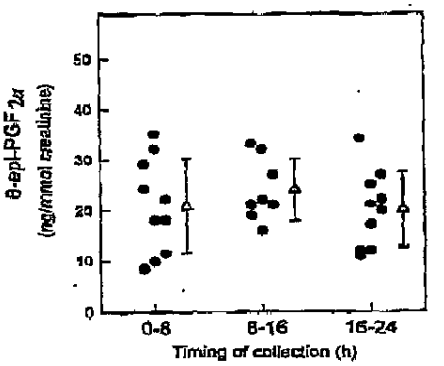

Fig. 5. Daily variation of urinary 8-epi-$PGF_{2\alpha}$ excretion analyzed in 10 healthy subjects. Consecutive 8-hr collections of urine were performed over 24 hr for each subjects. Collection and analysis of urines were done by EIA as described in "Materials and Methods." Open triangles represent mean ± S.D.

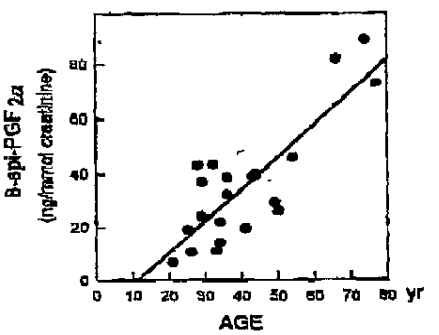

Fig. 6. Relationship of 8-epi-$PGF_{2\alpha}$ excretion with age in 20 subjects ($r = 0.81$; $P < 0.001$). Analysis of the samples was done by RIA as described in "Materials and Methods."

$PGF_{2\alpha}$ during this period compared to the 2 days preceding administration (fig. 8). No measurement of 11-dehydro-TXB_2 was performed.

Recently, Pratico *et al.* (1995) reported that human platelets can also generate small amounts of 8-epi-$PGF_{2\alpha}$ by a cyclooxygenase-dependent mechanism. We have analyzed the formation of 8-epi-$PGF_{2\alpha}$ in the serum of six healthy subjects and found that small but significant levels of this compound could be detected. Under these conditions, very substantial amounts of TXB_2 are formed (fig. 9). It should be noted that the serum concentrations of 8-epi-$PGF_{2\alpha}$ are three to four orders of magnitude lower than those of TXB_2 (*i.e.*, pg *vs.* ng/ml). When serum of the same subjects was analyzed after aspirin intake, there was a 90 to 98% reduction of TXB_2 (*i.e.*, from 380 ± 200 to 7.6 ± 4 ng/ml, Mean ± SD) with a similar reduction in 8-epi-$PGF_{2\alpha}$ (from 87 ± 34 to 7 ± 2 pg/ml) (fig. 8A). To verify the specificity of 8-epi-$PGF_{2\alpha}$-like immunoreactivity detected by direct assay measurement, we performed the analysis of the same samples before and after aspirin (*i.e.*, at high and low concentrations) after extraction and TLC purification; correlation between values was $r = 0.91$, $n = 10$, $P < 0.001$. The amounts of 8-epi-$PGF_{2\alpha}$ present in serum, before or after aspirin, were correlated with the amounts of TXB_2 ($r = 0.89$, $n = 10$, $P < 0.0001$) (fig. 8B). Altogether, these results suggest that the minute amounts of 8-epi-$PGF_{2\alpha}$ formed *ex vivo* during whole blood clotting are largely dependent on platelet cyclooxygenase activity.

Discussion

The discovery of a new family of biologically active arachidonic acid derivatives, *i.e.*, F_2-isoprostanes formed via non-enzymatic mechanisms, has rendered necessary the development of new assays to establish their formation and importance in pathological situations involving oxidative stress. We report obtention of antibodies against 8-epi-$PGF_{2\alpha}$ and development of immunoassays for this compound. Both RIA and EIA appear to provide good sensitivity and specificity although we could not test further cross-reactivities with other isoprostanes, due to the lack of appropriate standards.

Direct analysis of eicosanoids in urine by immunoassays is not possible as previously discussed for other arachidonic acid metabolites (Patrono, 1987). Extraction and purification of the compounds are a prerequisite to their analysis because

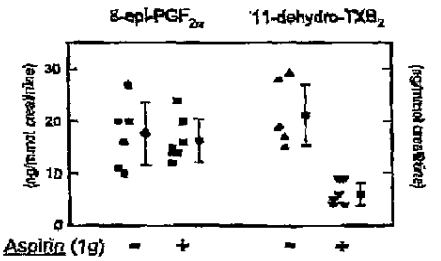

Fig. 7. Effects of aspirin on the urinary excretion of 8-epi-$PGF_{2\alpha}$ in healthy volunteers. Left of panel, urinary excretion 8-epi-$PGF_{2\alpha}$ before (●) and after (■) administration of a single dose (1 g) of aspirin in six healthy subjects. Right of panel, urinary excretion of 11-dehydro-TXB2 before (▲) and after (▼) aspirin treatment. Each subject ingested 1 g of aspirin at 6 P.M. and urines were collected from midnight in three consecutive 8-hr fractions. Analyses of 8-epi-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 were done by EIA as described in "Materials and Methods." Because the values remained unchanged (*i.e.*, <15% of variation) in the fractions, we have represented the mean of the three determinations for each subject.

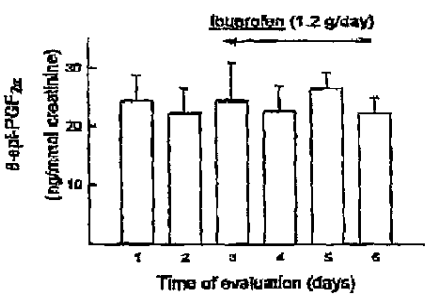

Fig. 6. Effects of daily intake of Ibuprofen (1.2 g/day for 4 days) on 8-epi-$PGF_{2\alpha}$ urinary excretion in four healthy subjects. Analysis of 8-epi-$PGF_{2\alpha}$ was done by RIA as described in "Materials and Methods."

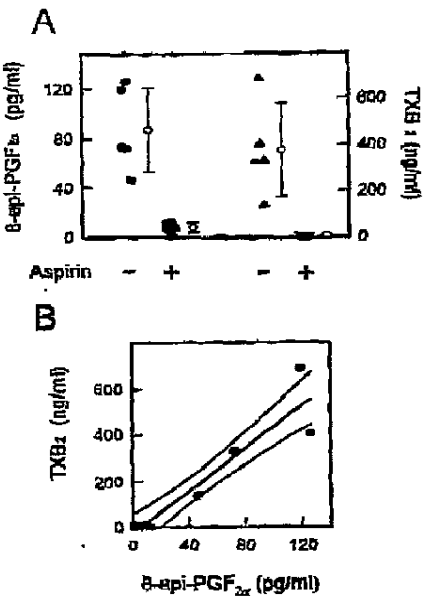

Fig. 8. Effects of aspirin on serum levels of 8-epi-$PGF_{2\alpha}$ and TXB_2 in healthy subjects. A single oral dose (1 g) of aspirin was administered to six subjects (same as in fig. 5) and serum was collected from five subjects as described in "Materials and Methods," before and 12 hr after dosing. A, Left of panel, 8-epi-$PGF_{2\alpha}$ levels in serum before and after aspirin. Right of panel, corresponding levels of TXB_2 in the same samples. Note the difference in scale (pg/ml for 8-epi-$PGF_{2\alpha}$ vs. ng/ml for TXB_2). B, Correlation between 8-epi-$PGF_{2\alpha}$ and TXB_2 in the different samples of serum (*i.e.*, before and after aspirin ingestion). TXB_2 and 8-epi-$PGF_{2\alpha}$ were analyzed directly by EIA. Similar values were obtained when serum samples were purified by TLC after solid-phase extraction as described in "Materials and Methods."

of interference by structurally related metabolites and other materials. The specificity of immunological measurements was validated by comparing values that had been obtained by GC/MS with those measured by TLC/EIA. A very good correlation was obtained between these two techniques. The extraction/purification procedures required prior to either EIA or RIA, albeit different for each technique, resulted in similar values for the urinary excretion of 8-epi-$PGF_{2\alpha}$ (see figs. 6 and 7). Either chromatographic method of purification (*i.e.*, silicic acid column or plate) before the immunoassays can be used. Additionally, measurement of the same urine extracts by different antisera possessing distinct specificities also gave very similar results. The use of [^{3}H]-TXB_2 and the purification protocol are identical to the procedure already used for EIA of other urinary metabolites (Lellouche *et al.*, 1990). Therefore, this protocol provides the opportunity to analyze an additional metabolite from the same purified extract. Because of its sensitivity, EIA could be used successfully for direct measurement of 8-epi-$PGF_{2\alpha}$ in serum since values (low and high) obtained after purification were similar to those obtained by direct measurement (see comments of fig. 8). Additional measurements of 8-epi-$PGF_{2\alpha}$ in plasma should be performed to assess the usefulness of measuring this compound in various diseases associated with a modification of the peroxidation tone and/or during generation of free radicals. It should be emphasized that *ex vivo* formation of isoprostanes can result from autoxidation (Morrow *et al.*, 1990a). Immediate processing of the samples and/or adding antioxidant and storage at −70°C appear to reduce this artefactual formation because we did not observe an increase of immunoreactive material up to 6 mo under these conditions.

Although the rationale for measuring PGF_2-isoprostanes in various pathological conditions has been discussed extensively (Morrow *et al.*, 1990b, Lynch *et al.*, 1994, Roberts and Morrow, 1995), specific assessment of 8-epi-$PGF_{2\alpha}$ production has received little attention. We have found that human volunteers present a highly reproducible rate of excretion of 8-epi-$PGF_{2\alpha}$ and that circadian variation is not detectable. Excretion of this compound was significantly elevated in the urines of two heavy smokers (*i.e.*, >20 cigarettes/day), (data not shown) compared to other normal individuals consistently with the recent report of Delanty *et al.* (1994). The enhanced excretion of endogenous excretion of 8-epi-$PGF_{2\alpha}$ in older subjects is consistent with an earlier report showing an elevation of arachidonic acid metabolites in apparently healthy older subjects (Reilly and FitzGerald, 1986) and will deserve further investigation. Urinary excretion of 8-epi-$PGF_{2\alpha}$ was not modified by the administration of two structurally unrelated cyclooxygenase inhibitors as anticipated

from its noncyclooxygenase origin. Under the same conditions, the urinary excretion of platelet cyclooxygenase-derived 11-dehydro-TXB_2 was largely suppressed in agreement with the known effect of aspirin on TXA_2 biosynthesis *in vivo* (FitzGerald et al., 1983). More unexpected was the formation of 8-epi-$PGF_{2\alpha}$ as a cyclooxygenase-dependent metabolite found in the serum of normal individuals. Its synthesis in relation to TXA_2 was reinforced by parallel suppression after aspirin intake evaluated by *ex vivo* measurement of TXB_2 and 8-epi-$PGF_{2\alpha}$. The formation of this compound has already been reported as a minor by-product of PGH synthase (Hecker *et al.*, 1987). Similar observations have recently been reported in platelets (Pratico *et al.*, 1995). Moreover, induction of PGH synthase-2 in human monocytes is associated with cyclooxygenase-dependent generation of 8-epi-$PGF_{2\alpha}$, that can be suppressed by selective inhibitors of this inducible enzyme (Patrignani *et al.*, 1995). Our finding that neither ibuprofen nor aspirin affected the urinary excretion of 8-epi-$PGF_{2\alpha}$ to any detectable extent in healthy subjects strongly suggests that such enzymatic mechanisms of isoprostane biosynthesis do not contribute importantly to the global biosynthesis of 8-epi-$PGF_{2\alpha}$ *in vivo* under physiological circumstances. However, the potential for a cyclooxygenase-independent component in isoprostane biosynthesis should be considered in the setting of platelet and monocyte activation and rigorously tested with adequate pharmacological tools.

Our study was designed to use immunoassays of 8-epi-$PGF_{2\alpha}$ to establish the patterns of excretion of this compound in healthy humans. The vasoconstrictor activity of 8-epi-PG isomers might play a deleterious rule in pathological states such as ischemia/reperfusion (Delanty *et al.*, 1994). Our results constitute a broad basis for the analysis of F_2-isoprostane biosynthesis in diseases such as vascular occlusive disorders where free radical generation and oxidative modifications of low density lipoproteins are thought to be of importance (Lynch *et al.*, 1994). It is possible that this approach will facilitate the rational evaluation of antioxidant drugs in humans. Perhaps more importantly, such compounds might provide stable markers of oxidative reactions in humans, as they possess longer half-lives than hydroperoxides and/or reactive oxygen species (Roberts and Morrow, 1995).

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